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PATENT APPLICATION 1029

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application Of:	)	
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Johanne TREMBLAY, *et al.*	)	Attorney Docket No. 04780.00001
	)	
Serial No.: 09/904,568	)	Group Art Unit: TBA
	)	
Filed: July 16, 2001	)	Examiner: Not Yet Assigned

For: HCaRG, A NOVEL CALCIUM-REGULATED GENE CODING FOR A NUCLEAR PROTEIN

SUBMISSION OF PRIORITY DOCUMENT

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Submitted herewith is a certified copy of Canadian Patent Application No. 2,312,266 filed in Canada on July 14, 2000. This application is the basis for Applicants' claim for priority, which claim was made upon filing of the above-identified patent application on July 16, 2001.

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Respectfully submitted,

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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,312,266**, on July 14, 2000, by **CENTRE DE RECHERCHE DU CENTRE HOSPITALIER DE L'UNIVERSITÉ DE MONTRÉAL**, assignee of Johanne Tremblay, Pavel Hamet, Nicolas Sölban and Richard Lewanczuk, for "The Use of HCaRG, a Novel Calcium-Regulated Gene Coding for a Nuclear Protein, for Regulating Cell Proliferation".

Agent certificateur/Certifying Officer

July 25, 2001

Date

Canada

(CIPO 68) 01-12-00

OPIC CIPO

TITLE OF THE INVENTION:

The Use of HCaRG, a Novel Calcium-Regulated Gene Coding for a Nuclear Protein for Regulating Cell Proliferation

BACKGROUND OF THE INVENTION:

Ionized calcium concentration in plasma is maintained within a very narrow range. The major players maintaining extracellular calcium homeostasis are calciotropic hormones, parathyroid hormone (PTH),[1] 1,25 dihydroxyvitamin D, calcitonin and calcium itself. Indeed, extracellular calcium regulates its own concentration as an extracellular messenger by acting on calcium receptors or calcium sensors. The calcium-sensing receptor is linked to several intracellular second messenger systems via guanylyl nucleotide-regulating G proteins and activates phosphoinositide-specific phospholipase C, leading to accumulation of inositol 1,4,5 trisphosphate (IP3) and diacylglycerol (1-5).

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[1]List of abbreviations

BN.lx	Brown-Norway rats
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
FCS	Fetal calf serum
GFP	Green fluorescent protein
GST	Glutathione S-transferase
HCaRG	Hypertension-Related, Calcium-Regulated Gene
IP3	Inositol 1,4,5 trisphosphate
MTE	Multiple tissue expression
nCARE	Negative calcium-responsive element
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PHF	Parathyroid hypertensive factor
PTC	Parathyroid cells
PTH	Parathyroid hormone
RACE	Rapid amplification of cDNA ends
RT	Reverse transcription
SDS	Sodium dodecyl sulfate
SHR	Spontaneously hypertensive rat
SSC	Standard sodium citrate
VSMC	Vascular smooth muscle cells
WKY	Wistar-Kyoto rats
ZFP7	Zinc finger protein 7

Cells of the parathyroid gland possess such a calcium sensor (6). Even slight reductions in extracellular ionized calcium concentration (in the order of 1-2% or less) elicit prompt increases in the rate of PTH secretion and mRNA levels. Historically, research on the parathyroid gland has focused on the chemistry, regulation, synthesis and secretion of PTH. There is growing interest in other calcium-regulating proteins of this gland that are also negatively regulated by extracellular calcium, such as chromogranin A and Secretory Protein-I (7), as well as a hypertensive factor of parathyroid origin (PHF) (8,9).

Arterial hypertension is associated with numerous disturbances of calcium metabolism manifested not only in humans but also in genetic as well as acquired models of hypertension (10-14). Disturbances in renal and intestinal handling of calcium in hypertension have been reported by several investigators (15). Urinary calcium has generally been shown to be increased (so-called urinary leak) and intestinal calcium absorption diminished in genetically hypertensive or spontaneously hypertensive rats (SHR) (15,16). Cytoplasmic free calcium concentration has most often been found to be elevated in circulating platelets, lymphocytes, erythrocytes, and vascular smooth muscle cells (VSMC) from hypertensive animals and humans (for review, see (17)). In SHR as well as in low-renin hypertensive patients, there seems to be an inverse relationship between extracellular and intracellular calcium (18). It has been hypothesized that certain genetic abnormalities might be responsible for the link between some forms of hypertension, calcium homeostasis and the parathyroid gland. To identify new genes that might be abnormally regulated by extracellular calcium in the parathyroid gland of genetically hypertensive rats, we prepared a cDNA library from the parathyroids of SHR. In this study, we describe the isolation and characterization of a novel gene, designated *HCaRG* (for Hypertension-related, Calcium-regulated Gene), negatively regulated by extracellular calcium with higher mRNA levels in SHR. *HCaRG* is a nuclear protein with putative 'leucine zipper' motifs and is potentially involved in the regulation of cell proliferation.

EXPERIMENTAL PROCEDURES

Cell cultures

Parathyroid cells (PTC) were isolated from SHR and Wistar-Kyoto (WKY) rats. Primary cultures were passaged in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS), as described previously (9). They were then maintained in Ham F12 medium containing a low (0.3 mM) or normal (2.0 mM) total calcium concentration for 2 or 48 h. COS-7 or HEK293 cells were cultured in DMEM containing 10% fetal calf serum. All cell types were maintained in 5% CO_2 at 37°C.

Ischemia-reperfusion

SHR were anesthetized with light flurane, and the right kidney was removed through a mid-abdominal incision. The left kidney was subjected to warm transient ischemia by occlusion of the left renal artery and vein with a micro-clip, as described previously (19). The skin incision was temporarily closed. After 60 min of occlusion, the clip was removed, and the wound was closed with a 2-0 suture. The rats had access to water immediately after surgery.

SHR parathyroid cDNA library

Parathyroid glands were removed from 100 12-week-old SHR and frozen immediately in liquid nitrogen. The glands were added to a guanidinium thiocyanate solution and homogenized. Poly A RNA was isolated on an oligo(dT) column. The cDNA library was constructed with Poly A RNA as template and the ZAP-cDNA synthesis kit (Stratagene, La Jolla, U.S.A.). A summary of the protocol is as follows: mRNA was reverse-transcribed from an XhoI-linker oligo(dT) primer using Moloney-Murine leukemia virus reverse transcriptase. Second strand synthesis was then produced with DNA polymerase I in the presence of RNaseH. cDNA termini were blunted by incubation with the Klenow fragment of DNA polymerase I and dNTPs. EcoRI adaptors were added using T4 ligase, and the ends phosphorylated with T4 polynucleotide kinase. This mixture

was then digested with Xho I to release adaptors from the 3' end of the cDNA. The resulting mixture was separated on a Sephacryl S-400 column. cDNAs were ligated into the Uni-ZAP XR vector using T4 DNA ligase, and packaged into Gigapack II Gold packaging extract. The packaged products were plated onto XL1-Blue MRF'. To screen the cDNA library, phages were plated onto bacterial host plates (XL1-Blue MRF') and incubated overnight. After chilling at 4°C for 2 h, a nitrocellulose filter was overlaid for 2 min. The filter was then denatured, neutralized and DNA crosslinked to it with UV light. Hybridization was performed with digoxigenin-dUTP labeled probes (Roche Molecular Biochemicals, Laval, Canada) derived from 3'- and 5'-RACE (rapid amplification of cDNA ends), products described below.

RNA and cDNA preparation

Total RNAs were prepared from rat cells and organs according to the standard guanidinium thiocyanate-phenol-chloroform method (20) and kept at -70°C until used. mRNA was extracted from total RNA with the PolyATtract system (Promega, Nepean, Canada). cDNAs, unless stated, were synthesized with random hexamers for first strand synthesis and reverse-transcribed. Radiolabeled DNA probes were prepared by the random priming technique or polymerase chain reaction (PCR) amplification with ^{32}P-dCTP.

3' or 5'-RACE

Four mixtures of degenerate oligonucleotide primers were initially designed according to the putative amino acid sequence of PHF with the following degenerate sequence: 5' TA(T/C) TCI GTI TCI CA(T/C) TT(T/C) (A/C) G 3'. From initial RACE experiments (described below), 1 unique sequence primer TAC TCC GTG TCC CAC TTC CG was selected for its ability to generate reverse transcription (RT)-PCR DNA fragments from PTC total RNA and used subsequently as candidate primer for 3'-RACE. In brief, for 3'-RACE, total RNA from PTC was reverse-transcribed with a hybrid primer consisting of oligo(dT) (17 mer) extended by a unique 17-base oligonucleotide (adaptor). PCR amplification was subsequently performed with the

adapter, which bound to cDNA at its 3'-ends, and the candidate primer mentioned above (21). For 5'-RACE, RT was undertaken with an internal primer derived from the sequence of the cDNA fragment generated by 3'-RACE. A dA homopolymer tail was then appended to the first strand reaction products using terminal deoxynucleotidyl transferase. Finally, PCR amplification was accomplished with the hybrid primer described previously and a second internal primer upstream to the first one (21).

Subcloning

The DNA fragments generated from the RACE experiments were separated by electrophoresis, isolated from agarose gel and extracted by the phenol-chloroform method (20). pSP72 plasmid (Promega) was digested at the Sma I site and ligated to blunt DNA fragments with T4 DNA ligase. Transformed DH5α *E. coli* bacteria were grown and recombinant bacteria were selected by PCR. Similarly, *HCaRG* was subcloned in pcDNA1/Neo (Invitrogen, Carlsbad, U.S.A.).

To determine the subcellular localization of *HCaRG* protein in mammalian cells, the coding region of *HCaRG* was fused to green fluorescent protein (GFP) cDNA and was transfected in the cells. Briefly, the entire coding region of *HCaRG* was amplified by PCR with the primers ATG TCT GCT TTG GGG GCT GCA GCT CCA TAC TTG CAC CAT CCC and TAA TAC GAC TCA CTA TAG GGA GAC, gel purified, and fused in-frame to GFP in pEGFP-C1 (Clontech, Palo Alto, U.S.A.) through a blunt Hind III site. pEGFP-*HCaRG* was then sequenced. Similarly, the coding sequence of *HCaRG* was fused in frame to glutathione S-transferase (GST) in pGEX-3X (Amersham Pharmacia Biotech, Baie d'Urfée, Canada) through a Sma I site and a blunt EcoR I site.

Sequencing

Double-stranded sequencing of cloned cDNA inserts was performed with Sequenase Version 2.0 (United States Biochemical, Cleveland, U.S.A.). 5 μg of recombinant plasmid template were

denatured, annealed with T7 or SP6 primers, and labeled with ^{35}S-dATP by extension, using the chain termination method of Sanger according to the manufacturer's protocol.

Cloning of human HCaRG

A 439-bp cDNA fragment of rat *HCaRG* was ^{32}P-labeled and served as a probe for screening a human VSMC cDNA library. DNA from positive phages was purified and the fragments were cloned in pBluescript. All fragments were sequenced. We obtained a 1355-bp fragment containing the coding region of *HCaRG*.

Northern blot hybridization, dot blot hydridization and competitive RT-PCR

2 µg of poly A RNA from PTC or 10 µg of total RNA from kidneys were denatured at 68°C and separated on denaturing formamide 1% agarose gel. After transfer onto nitrocellulose by vacuum, hybridization was performed overnight using ^{32}P-labeled probes generated from cDNA clone(s) by PCR or random labeling. 1 µg of total RNA was used in dot blot experiments. A human multiple tissue expression (MTE™) array (Clontech) and human fetal and tumor panel Northern Territory™ RNA blots (Invitrogen, Carlstad, U.S.A.) were hybridized with ^{32}P-labeled human *HCaRG* cDNA according to the manufacturer's specifications. For quantitative determinations of *HCaRG* mRNA, total RNA was extracted from PTC and reverse-transcribed. A *HCaRG* competitor was constructed using the PCR Mimic Construction Kit (Clontech) with the following composite primers: GCA CGA GCC ACA GCC AGC TAC CCC AGC CAC CCA TTT GTA CC (sense) and TGT GAC TGT CAG CGG GAT GGA GTC CGA GAT GTA GAG GGC (antisense). The 344-bp DNA obtained was cloned into pSP72 and transcribed with SP6 RNA polymerase. The resulting RNA was quantified by photometry and subsequently used in competitive RT-PCR. The competitive reaction contained 1 or 2 µg total RNA with increasing amounts of competitor cRNA along with ^{32}P-labeled nucleotide. Two primers TGT GAC TGT CAG CGG GAT GG and GCA CGA GCC ACA GCC AGC TACC flanking the *HCaRG* intron were employed to amplify a 186-bp cDNA fragment. PCR was performed: 15 sec at 95°C, 20

sec at 68°C, 30 sec at 72°C, for 30 cycles, followed by a 5-min elongation step at 72°C. 10 µl of the PCR were loaded on 1.8% agarose gel, then dried and exposed in a PhosphorImager cassette for quantification.

In situ mRNA hybridization

Tissues from SHR and WKY rats were rinsed in phosphate buffer, fixed in 4% paraformaldehyde and embedded in paraffin. 3- to 5-µm sections were cut and mounted on microscope slides pretreated with aminopropylthiethoxysilane. The slides were first dried at 37°C, then at 60°C for 10 min prior to use. The probe applied was a unique 300-bp fragment (3r 290 in Figure 2A) which had been subcloned into the BamH I site of a pSP72 vector. The DNA was transcribed using T7 or SP6 polymerases to create sense and antisense riboprobes which were labeled with digoxigenin-UTP. They were validated by dot blot hybridization with template DNA. Prehybridization of slides was undertaken after de-waxing in xylene, followed by progressive ethanol-water hydration (95% to 50%). The slides were rinsed in phosphate-buffered saline (PBS) and incubated with proteinase K (20 µg/ml) for 20 min at room temperature. After this digestion, they were rinsed successively in glycine buffer, PBS and then dehydrated in ethanol. Actual prehybridization was done with 50% formamide, 0.2% sodium dodecyl sulfate (SDS), 0.1% Sarcosyl, 5X standard sodium citrate (SSC: NaCl (0.15M), sodium citrate (0.015M, pH 7.0)) and 2% blocking reagent (Roche Molecular Biochemicals) for 1 h at 60°C. Hybridization was performed by adding the probe (200 ng/ml) to 50 µl of 4X SSC and 50% formamide per section. The slides were incubated overnight at 60°C in a humidified chamber. During hybridization, a coverslip was placed over the tissue section. After hybridization, it was removed and the sections rinsed with 4X SSC, then washed with 4X SSC for 15 min and in 2X SSC for 15 min, at room temperature. Finally, the sections were washed with 0.1% SSC for 30 min at 60°C. For coloration, they were washed with Buffers 1 and 2 of the DIG Luminescent Detection Kit (Roche Molecular Biochemicals). They were then incubated with anti-DIG alkaline phosphatase antibody (1:500) in Buffer 2 for 40 min, washed twice in Buffer 1 for 15

min and in Buffer 3 for 2 min. Incubation in the color solution (NBT/x-phos) was carried out for 45 min, after which the slides were washed in distilled water and dry-mounted with Geltol.

In vitro translation

The full length of the *HCaRG* coding sequence was synthesized by RT-PCR with specific primers and inserted downstream of the T7 promoter into the pSP72 vector. *In vitro* transcription and translation were performed using a TNT-T7-coupled reticulocyte lysate system (Promega) in the presence of ^{35}S-methionine. A plasmid containing the luciferase gene supplied by the manufacturer was used as a control. The synthesized proteins were analyzed by 15% SDS-polyacrylamide gel electrophoresis (PAGE) in the absence or presence of ß-mercaptoethanol. Radioactive protein bands were detected by scanning with a PhosphorImager.

Antibody production

E. coli cells transformed with pGEX-3X were grown in LB medium containing 50 µg/ml ampicillin at 37^{0}C until $A_{595\ nm}$ = 0.5. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM, and the cells were cultured for 2 h. Purification of GST-*HCaRG* was performed according to the manufacturer's protocol. Polyclonal antisera with antibodies recognizing *HCaRG* were produced by immunization of rabbits with GST-*HCaRG* protein.

Immunocytological reaction at the electron microscopic level

Rat tissues (liver, anterior pituitary, spleen, heart and adrenal gland) were quickly removed and fixed in 4% paraformaldehyde with 0.05% glutaraldehyde in phosphate buffer solution for 90 min. A part of the specimens was cryoprotected in 0.4M sucrose phosphate buffer solution for 30 min at 4°C, then frozen in a cold gradient of fuming nitrogen (Biogel, CFPO, Saint Priest, France) to -4°C, and immersed in liquid nitrogen, as described previously (22). Ultrathin frozen

sections of 80 nm thickness were obtained using a dry sectioning method at -120°C with an Ultracut S microtome (Leica, Lyon, France). The other part of the specimens was dehydrated before embedding in Lowicryl K4M with the AFS system (Leica) (23). Sections were mounted on 400 mesh collodion-carbon-coated nickel grids. For ultrastructural localization of *HCaRG* protein, the grids were first placed in buffer containing 0.1 M phosphate buffer, 0.15 M NaCl, and 1% albumin, pH 7.4, for 10 min. They were then incubated for 1 h with polyclonal IgG raised against *HCaRG* protein at concentrations of 1:1000 and 1:50 for ultrathin frozen sections and Lowicryl sections respectively. After 10-min washing in the same buffer, antigen/antibody complexes were revealed with anti-rabbit IgG conjugated with 10 nm gold particles in buffer containing 0.05 M Tris, 0.15 M NaCl, 1% albumin, pH 7.6, for 1 h. The grids were washed in the same buffer and fixed with 2.5% glutaraldehyde. The specificity of the immunocytological reaction was tested on sections with omission of primary antibody and incubation of the primary antibody with particle-adsorbed antigen. No signal was observed on these tissue sections. Before observation in a Philips CM 120 electron microscope at 80 kV, the cryosections were contrasted in 2% uranyl acetate, embedded in 8% methylcellulose, and the Lowicryl sections were contrasted for 20 min in 5% uranyl acetate.

Transfection and subcellular localization

COS-7 cells were plated at ~30-50% confluency 1 day prior to transfection which was performed with 5 µg/well of pEGP-*HCaRG* or pcDNA1/Neo-*HCaRG*, according to the calcium phosphate method. After 24 h, the cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. Following 3 washes with PBS, cells transfected with pEGFP-*HCaRG* or pcDNA1/Neo-*HCaRG* were mounted on coverslips. The cells were permeabilized with 0.3% Triton X-100 for 12 min, blocked with 1% BSA-1% gelatin for 15 min, incubated with *HCaRG*

antibodies at 37°C for 1 h, washed in 0.5% BSA, incubated with anti-rabbit FITC-labeled antibodies and washed again. Fluorescence and immunofluorescence were detected with a Zeiss fluorescence microscope.

Stable transfection

HEK293 cells were plated in a 100-mm plate at a density of $0.5x10^6$ cells/plate. They were transfected with the control plasmid pcDNA1/Neo (Invitrogen, Faraday, U.S.A.) or with the plasmid containing rat *HCaRG* using a standard calcium phosphate coprecipitation method. 48 h after transfection, the cells were plated in 150-mm plates in the presence of 400 µg/ml G418 (Life Technologies, Burlington, Canada). After 2 weeks, the clones were picked and the level of ectopic *HCaRG* expression was determined by northern hybridization.

Cell counting and ^{3}H-thymidine incorporation

The rate of stable clone cell proliferation was measured by counting the number of cells after plating. Cells were seeded at a density of $0.1x10^6$ cells/6-well plate, with triplicate plates for each cell line. Every 24 h, the cells were trypsinized and counted in a hemocytometer. HEK293 cells which stably expressed either Neo control plasmid or *HCaRG* were used for the estimation of DNA synthesis by ^{3}H-thymidine incorporation. The clones were trypsinised at 90% confluency, counted in a standard hemocytometer and inoculated at an identical initial cell density of 40,000 cells/ml in DMEM containing 10% FBS and G418 at 400 µg/ml. All cells were inoculated in Poly-D-lysine-pretreated 24-well plates in a volume of 1 ml/well (40,000 cells/well). They were allowed to attach and grow for a period of 24-48 h. The growth media were then replaced by DMEM containing 0.2% FBS and G418 (400 µg/ml) for a period of 48 h to synchronise the cells. After the synchronisation period, the cells were supplied with fresh medium containing 10% FBS and allowed to grow for 48 h. [^{3}H]-thymidine, 1 µCi/ml (ICN) was added to the cells

for the last 4 h of the 48 h-growth period. At the end of incubation, the medium was removed and the monolayers washed twice with PBS. The cells were then fixed with ethanol:acetic acid (3:1, V:V), and DNA was digested/extracted with 0.5N PCA. at 80-90°C for 20 min.

RESULTS

Isolation of a novel cDNA whose expression is negatively regulated by extracellular calcium in the SHR parathyroid gland

Using sense candidate primers (from a putative amino acid sequence of PHF (24)) and a hybrid oligo dT primer, 3'-RACE experiments, performed on total RNA extracted from SHR PTC cultured in low-calcium medium, generated 1 major 700-bp fragment that was digested and cloned in the BamH I site of pSP72. As a BamH I site was present in the 700-bp fragment, a recombinant plasmid containing a 300-bp insert was isolated and sequenced. This fragment was used to screen the PTC library and to generate new oligonucleotide primers to extend the cDNA towards the 5'- and 3'-ends by RACE. From 7 overlapping DNA fragments isolated in the above experiments and from SHR PTC cDNA library screening, a 1100-bp cDNA was reconstituted (Fig. 1A). The rat 1100-bp reconstituted cDNA sequence contained an open reading frame of 224 codons preceded by 2 in-frame stop codons and followed by the most frequent variant of the poly A tail (Fig. 1B). A 342-bp intron was localized at position –52 from the translation initiation site.

Poly A RNA was isolated as described and analyzed by Northern hybridization with the ^{32}P-labeled 300-bp fragment (Fig. 2A). Two bands were detected with this probe, at approximate lengths of 1.2 and 1.4 kb. These results suggest either the existence of 2 genes or differential splicing. Furthermore, they indicate that the reconstituted 1100-bp cDNA is almost full length cDNA, estimated at 1.2 kb by the major band in the northern hydridization experiments.

Regulation of the expression of this novel gene was investigated by competitive RT-PCR assay in PTC from WKY and SHR. Cells between 5 and 12 passages were tested in these studies. In WKY PTC, lowering of ambient calcium from 2.0 mM to 0.3 mM induced a rapid 2-fold increase in the mRNA levels of this novel gene at 2 h, which lasted up to 48 h (Fig. 2B). This calcium regulation was detected in WKY PTC up to about 12 passages but disappeared in long-term cultures. Lowering of calcium concentrations in the cell medium also increased the mRNA levels of this novel gene in SHR PTC but to a lesser extent than in WKY cells (data not shown). We then compared its mRNA levels between 2 normotensive rat strains (Brown Norway, BN.lx, or WKY) and hypertensive animals (SHR). We observed that the mRNA levels of this novel gene were significantly higher in PTC derived from SHR (Fig. 2C left panel) compared to normotensive WKY rats at normal calcium. Similarly, when we extracted RNA (Fig. 2C right panel) or proteins (Fig. 2D) directly from the kidneys, we found significantly higher levels of this novel gene in hypertensive rats. These results clearly show that this novel gene is negatively regulated by extracellular calcium concentrations and that its levels are significantly higher in genetically hypertensive rats compared to 2 normotensive strains. We, therefore, named this gene Hypertension-related, Calcium-Regulated Gene (*HCaRG*).

Sequence and structure of HCaRG cDNA

The deduced protein contained 224 amino acids with a calculated molecular weight of 22456 Da. The estimated pI of the protein was 6.0. It comprised no known membrane-spanning motif but had an estimated 67% α-helix content. The absence of a putative signal peptide sequence suggested an intracellular protein. There were 2 cysteines in the sequence, indicating possible intra- or inter-molecular disulfide bridges (Cys 64-cys 218). The protein had several putative

phosphorylation sites for C- and A-kinases and 1 potential Asn-glycosylation site (Asn 76). To confirm that *HCaRG* mRNA encodes a peptide of expected size, the *HCaRG* cDNA inserted into pSP72 was incubated in vitro in a coupled transcription/translation labeling system. It was transcribed by T7 RNA polymerase, and translated in rabbit reticulocyte lysate. As shown in Figure 3 (lane 4), *HCaRG* mRNA directed the synthesis of a peptide with a molecular mass of 27 kDa which closely corresponded to the molecular weight calculated from the amino acid sequence. PAGE analysis of the reaction product in the absence of the reducing agent β-mercaptoethanol showed bands of 27 and 43 kDa (Fig. 3, lane 5). These results suggest possible intramolecular or intermolecular disulfide bridges and the formation of homodimers or heterodimers with other protein(s) present in the lysate.

Cloning of human HCaRG

We then used a 439-bp cDNA fragment of rat *HCaRG* (+1 to +440 in Fig. 1) to screen a human VSMC cDNA library. We identified several positive clones that were purified, subcloned in pBluescript vector and sequenced. We obtained a 1355-bp sequence containing full length human cDNA, while all other clones contained only partial sequences. A recent sequence search in GenBank revealed a region with complete DNA sequence homology within 3 cosmids containing the zinc finger protein 7 (ZFP7) gene (accession numbers AF124523, AF146367 and AF118808). Although the nucleotide sequence of human *HCaRG* could be found in these cosmids, we are the first to assign an expressed gene sequence to this DNA region.

Sequence comparison between human *HCaRG* and rat *HCaRG* showed 80% identity at the nucleotide level (data not presented) and, similarly, 80% homology at the amino acid level (Fig. 4). Analysis of protein structure with the PROSEARCH database revealed 4 overlapping putative

'leucine zipper' consensus motifs (Fig. 4 underlined). Further analysis revealed homology to the EF-hand calcium-binding motif (8 out of the 10 most conserved amino acids) (Fig. 4 dashed box). We also identified a nuclear receptor-binding motif (Fig. 4 bold and italics). All these motifs were conserved in the rat and human amino acid sequence.

Subcellular localization of HCaRG

We expressed GFP-*HCaRG* in COS-7 cells. Fluorescence study showed that GFP-*HCaRG* localized in the nucleus while cytoplasmic fluorescence was very faint (Fig. 5B). GFP, on the other hand, had a very diffuse localization (Fig. 5A). This result was confirmed by immunofluorescence using antibodies specific to *HCaRG* (Fig. 5C) and by electron microscopy (Fig. 5D). Electron microscopy was also performed on different tissues. In all tissues studied, *HCaRG* was found in the nucleus with some labeling in protein synthesis sites.

HCaRG expression in various human tissues

A human MTE™ array was hybridized with human ^{32}P-labelled *HCaRG* cDNA as a probe. The array contained 76 polyA RNAs from various adult tissues, cell lines, fetal tissues and cancerous cell lines. These arrays were normalised to 8 different housekeeping genes. Analysis of the array showed that *HCaRG* was expressed preponderantly in the heart, stomach, jejunum, kidney, liver and adrenal glands. Comparison of *HCaRG* expression in fetal to adult organs revealed that *HCaRG* mRNA was less expressed in all fetal tissues compared (Fig. 6A), particularly in the heart, kidney and liver. Northern blots confirmed the lower abundance of *HCaRG* in the fetal heart compared to all regions of the adult heart (Fig. 6B). We also compared *HCaRG* mRNA levels in various cancerous cell lines to normal tissues (Fig. 6C). *HCaRG* mRNA levels were decreased in all cancerous cell lines studied. They were also much lower in a glioblastoma, a

partly-differentiated renal cell carcinoma and a moderately differentiated hepatocellular tumor compared to the same amount of normal RNA of adjacent tissues excised from the same operational site (Fig. 6D).

In situ hybridization of HCaRG mRNA in the kidney and adrenal

HCaRG expression was determined in SHR tissues by *in situ* hybridization. The labeled antisense riboprobe hybridized to the medulla and zona fasciculata of the adrenal cortex (Fig. 7). In the kidney, labeling was almost exclusively located in the cortex and concentrated in the tubular component, contrasting with virtual absence of the signal in glomeruli (Fig. 7). In these organs, the signal was clearly greater in hypertensive rats compared to their normotensive controls (Lewanczuk et al.; unpublished data). The sense probe was used as a negative control and appropriately revealed a low signal under our hybridization conditions, demonstrating specificity of the reaction (Fig. 7 lower panels).

HCaRG mRNA levels after ischemia-reperfusion

The process of kidney injury and repair recapitulates many aspect of development. It involves de-differentiation and regeneration of epithelial cells, followed by differentiation (25-27). Since we observed that *HCaRG* mRNA levels are lower in fetal than in adult organs, we evaluated *HCaRG* expression after unilateral renal ischemia in uninephrectomized rats (19) as contralateral nephrectomy has been shown to stimulate cell regeneration (28-31). We noted that *HCaRG* mRNA declined rapidly to its lowest levels at 3 h and 6 h of reperfusion (Fig. 8A). These values then increased steadily to higher than baseline at 48 h of reperfusion. This was observed in both the kidney medulla (Fig. 8A) and cortex (Fig. 8B). In contrast to the decline in *HCaRG* mRNA

levels, the proto-oncogene *c-myc* expression, which is correlated with hyperplastic response in mammalian cells, was rapidly increased following renal ischemia and reperfusion (31). *c-myc* mRNA levels were low in control kidneys and increased dramatically in the post-ischemic kidney at 3 h of reperfusion, at a time when *HCaRG* mRNA levels were already reduced (Fig. 8A and 8C)

Overexpression of HCaRG inhibits cell proliferation

HEK293 cells were stably transfected with either plasmid alone or with plasmid containing rat *HCaRG*. After transfection, several clones were examined for the determination of rat *HCaRG* mRNA levels. Four clones (*HCaRG* clones 1, 5, 8 and 9) expressed variable amounts of rat *HCaRG* mRNA, as detected by northern blots, while no *HCaRG* mRNA levels were found in clones transfected with the plasmid alone (Fig. 9). Clones expressing the highest levels of *HCaRG* (clones 8 and 9) were selected for cell proliferation studies. For these studies, cells that were transfected with the vector alone or polyclonal *HCaRG*-transfected cells served as controls. The proliferation rates of the *HCaRG*-transfected cell lines and vector control cells were examined under normal growth conditions (10% FCS and G-418) by counting cell numbers every day for a period of 8 days after plating. Cell lines transfected with the vector alone (Neo clones 1 and 6) showed a similar growth rate as non-transfected cells (not presented). Clones 8 and 9 expressing high levels of rat *HCaRG* revealed a much lower proliferation rate than vector control cells while polyclonal cells expressing intermediate values of *HCaRG* fell in between (Fig. 10A). Consistent with a lower proliferation rate, stable *HCaRG* transfection clones 8 and 9 showed much lower ^{3}H-thymidine incorporation than clones transfected with the empty vector (Fig. 10B).

DISCUSSION

The cloning of a novel extracellular calcium-responsive gene (*HCaRG*) in the rat parathyroid gland from SHR is described here. *HCaRG* mRNA and protein levels were higher in cultured PTC and in several organs of SHR, compared to their normotensive counterparts. They were negatively regulated by extracellular calcium, i.e. lowering extracellular calcium led to increases in *HCaRG* mRNA. The identification of an extracellular calcium-sensing receptor from the parathyroid gland has provided novel insights into the mechanisms of direct action of extracellular calcium on several cell types. The calcium sensor has also been localized in the cerebral cortex and cerebellum, in the tubular region of the kidney cortex, the thyroid, adrenal medulla, lung, and blood vessels (1,32,33). As shown here, *HCaRG* mRNA levels are also detected in several of these tissues. The calcium receptor is a member of the superfamily of G protein-coupled receptors activating phospholipase C (34,35). In the parathyroid gland, it is a key mediator of inhibition of PTH expression by high calcium (36). The calcium sensor has been shown, in the kidney, to be directly related to inhibition of tubular reabsorption of calcium and magnesium in the thick ascending loop (for review, see (34)). In PTC cultures prepared from human or bovine parathyroids, low extracellular calcium (0.3 mM) has been demonstrated to increase PTH secretion and mRNA levels whereas augmentation of calcium in the incubation medium reduces PTH mRNA. Similar regulation was observed for PHF in rat parathyroid cells (9). We show here that *HCaRG* expression is regulated in a manner similar to PTH and PHF in PTC isolated from the rat.

To date, very few extracellular calcium-negative responsive genes have been cloned. Parathormone was the first gene described to possess a negative calcium-responsive element (nCARE) in its 5'flanking region (37). Several types of nCARE have been reported: Type 2 is a regulatory element consisting of a palindromic core sequence and several upstream T nucleotides

originally described in the PTH gene. Its transcriptional inhibitory activity is orientation-specific. The nCARE core is present in an Alu-repeat in 111 copies in the human genome, suggesting the possibility that other genes may possess functional nCARE (38). With the properties described in the present study, *HCaRG* may be one of them.

HCaRG is not only expressed in the parathyroid gland but also in most organs tested, although at highly variable levels. Elevated *HCaRG* levels have been noted consistently in the tissues of genetically hypertensive animals, suggesting abnormalities of *HCaRG* regulation in several organs of SHR that could be due to either: 1) decreased extracellular calcium levels; 2) an abnormal response to extracellular calcium; 3) abnormal transcription/stability of *HCaRG* mRNA in hypertensive rats, or 4) a combination of these. A state of negative calcium balance has been described in SHR that could support the first possibility. On the other hand, 2-fold higher *HCaRG* mRNA levels were observed in PTC from SHR than from WKY at normal calcium concentration (Fig. 2C). Thus, the modest reduction of calcemia in hypertension will not be the sole explanation of increased levels, suggesting increased expression or decreased degradation of this gene product in hypertension.

No homologous protein sequence to the *HCaRG* open reading frame was found in the SWISSPROTEIN database. The *HCaRG* coding sequence contains 1 consensus motif known as the EF-hand or HLH Ca motif (Fig. 3 dashed box). This motif generally consists of a 12-residue, Ca-binding loop flanked by 2 α-helices. Eight of the 10 most conserved amino acids are present in *HCaRG* protein. Usually, the basic structural/functional unit consists of a pair of calcium-binding sites rather than a single HLH motif. The *HCaRG* coding sequence contains only 1 EF-like motif but it is possible that its high α-helix content favours coiled-coil interactions and dimerization of the protein. Pairing of the 2 EF-hand motifs may enhance its calcium function. Hodges and collaborators (39,40) have demonstrated that domain III of troponin C (a synthetic

34-residue calcium-binding domain) can form a symmetric 2-site homodimer in a head-to-tail arrangement in the presence of calcium (41). Similarly, a 39-residue proteolytic fragment containing calcium-binding site IV of troponin C was shown to form a dimer (42). These studies and others (43-45) have demonstrated that dimerization of single HLH structures controls calcium affinity and that even homodimers can bind 2 calcium molecules with positive cooperativity (40). Hydrophobic interactions at the interface between calcium-binding sites appear to stabilize the calcium domains. Our in vitro translation studies showed the appearance of a protein band of about 43 kDa under non-reducing conditions. *HCaRG* protein might form reductant-sensitive, non-covalent homodimers compatible with its putative high α-helix content, but the existence of a functional calcium domain in *HCaRG* protein remains to be established. Several characteristics of *HCaRG* are similar to those of S100A2 protein, a calcium binding protein of the EF-hand type that is preferentially expressed in the nucleus of normal cells but down-regulated in tumors (44). As with *HCaRG*, S100A2 expression is down-regulated by calcium (46,47).

We also cloned the human homolog of *HCaRG* from a VSMC cDNA library, using a 437-bp fragment of rat *HCaRG* as a probe. The coding sequence was found to be 80% homologous to the rat sequence and to contain the putative EF-hand domain. A restriction fragment length polymorphism permitted us to localize the *HCaRG* locus on chromosome 7 of rats (Tremblay et al, unpublished). The gene was assigned within a 4.4-cM region on the long arm of chromosome 7 between Mit 3 and Mit 4 genes. By analogy, we suggested the assignment of *HCaRG* on human chromosome 8q21-24. In a recent search of *HCaRG* homologous sequences in Genbank, homologies were found with 3 chromosome 8 clones containing ZFP7. It was, therefore, possible to localize *HCaRG* on chromosome 8q24.3, confirming our initial assignment. This region contains loci involved in several bone diseases, including osteopetrosis and multiple exostosis and several human neoplasms (48,49).

Many DNA-binding proteins utilize zinc-containing motifs to bind DNA. Other classes of DNA-binding proteins have a DNA-recognition domain at their N terminus that dimerizes to form a 2-chain coiled-coil of α-helices, also known as a 'leucine zipper'. We identified 4 overlapping 'leucine zipper' regions conserved in the rat and human sequence, and the high α-helix content of *HCaRG* makes it a possible DNA-binding protein. We are currently investigating this possibility. It has been shown that nuclear receptors require the ligand-dependent recruitment of co-activator proteins to effectively stimulate gene transcription (50). The nuclear receptor interaction domain of these factors is highly conserved and contains the consensus sequence LXXLL (where X is any amino acid). This motif is sufficient for ligand-dependent interaction with nuclear receptors (51). We have identified 1 of these motifs in *HCaRG*. Nuclear localization of *HCaRG* protein makes this gene a potential transcription regulator.

Recently, a new transcription factor from the rat kidney (Kid-1) was identified (52-55). It was reported that Kid-1 mRNA levels declined after renal injury secondary to ischemia (55). Similarly, decreased *HCaRG* mRNA levels are seen when epithelial cells are de-differentiated and proliferate (following ischemia and reperfusion). In the model of unilateral ischemic injury, it was shown that contralateral uninephrectomy attenuates apoptotic cell death and stimulates tubular cell regeneration (28-31). We demonstrate here that *HCaRG* mRNA levels decreased 3 and 6 h after ischemia in contrast to c-myc expression which is correlated with hyperplastic responses (31). We also observed that its levels are lower in all fetal organs tested when compared to adult organs, and lower in tumors and the cancerous cell lines tested. It is possible that the gene product may exert a negative effect on growth. This was confirmed by the stable expression of *HCaRG* in HEK293 cells. We found that *HCaRG* overexpression had a profound inhibiting effect on HEK293 cell proliferation. This was shown not only by lower cell number

but also by lower DNA synthesis, suggesting that the effect seen was not due to a death-promoting effect of *HCaRG*.

In conclusion, we have cloned the cDNA of a novel gene that is regulated negatively by extracellular calcium and presents greater expression in several organs of the genetically hypertensive rat model which is known to demonstrate negative calcium balance. *HCaRG* mRNA levels are rapidly regulated by calcium, perhaps via the action of calcium receptor signaling. Comparison of *HCaRG* mRNA levels in fetal to adult organs and normal and tumour cells showed that *HCaRG* is more expressed in all adult normal tissues tested. We also report that *HCaRG* mRNA levels are modulated during ischemia-reperfusion injury which mimics kidney ontogeny. Furthermore, its nuclear localisation, identified motifs and patterns of expression make this gene a potential regulator of cellular proliferation.

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FIGURE LEGENDS:

FIGURE 1. cDNA cloning of *HCaRG*. *A*. Reconstitution scheme of *HCaRG* cDNA. Overlapping fragments leading to the reconstitution of rat *HCaRG* 1100-bp cDNA are shown. cDNA fragments were initially obtained using 5'-RACE and 3'-RACE strategies as well as by screening a SHR parathyroid cDNA library. The first cDNA fragment was by 3'-RACE (3r 290). This initial fragment served to screen the SHR parathyroid cDNA library. Fragments *HCaRG* 2c-t3 + 2c-t7, *HCaRG* 825, *HCaRG* 10-ic, and *HCaRG* 10-174 were isolated from the cDNA library. Fragments 5r 285 and 5r 260 were obtained by 5'-RACE. This reconstitution was confirmed by sequencing a 860-bp PCR product with nested primers in 5r 260 and *HCaRG* 825 and containing the complete open reading frame. *B*. Nucleotide and deduced amino acid sequences of *HCaRG*. The translation initiation start site codon is at position 1 and the termination codon is at position 675. The deduced amino acids are indicated below the nucleotide sequence. The localization of a 482-bp intron is indicated at position –52 by a triangle.

FIGURE 2. Identification of a novel gene negatively regulated by extracellular calcium. *A*. Northern blot analysis of Poly A RNA isolated from parathyroid cells (PTC). *HCaRG* mRNA appears as a doublet of approximately 1.2 and 1.4 kb. The positions of ribosomal RNAs and GAPDH transcript are indicated. *B*. PTC extracted from normotensive rats (WKY) (from passages 8 to 10) were incubated in low (0.3 mM) or normal (2 mM) calcium-containing medium for 2 and 48 h. Total RNA was extracted and analysed by RT-PCR as described in the Experimental Procedures section. Incubation of PTC for 2 h in 0.3 mM (L) calcium significantly increased *HCaRG* mRNA compared to 2 mM (N) calcium; this increase lasted up to 48 h. *C*.

Significantly higher basal *HCaRG* levels were found in PTC from hypertensive rats compared to the normotensive rat strain WKY (left panel). This was confirmed with RNA (right panel) and proteins (*D*) extracted directly from the kidneys of SHR and BN.*lx*, another normotensive strain. The figure represents the mean ± S.E.M. of 2 independent experiments performed in duplicate. ** indicates $p<0.02$, * indicates $p<0.05$ as evaluated by the unpaired *t*-test.

FIGURE 3. In vitro translation of *HCaRG* cDNA. cDNA was cloned into pSP72 vector and used for coupled transcription/translation in the presence of ^{35}S-methionine. Lane 1: molecular weight markers; lane 2: translation products of the control luciferase gene; lane 3: translation products without the insert; lane 4: translation product from *HCaRG* cDNA; lane 5: translation products of *HCaRG* cDNA. The proteins were separated by 15% PAGE in the presence (lanes 1 to 4) or absence (lane 5) of β-mercaptoethanol. Transcription/translation of *HCaRG* cDNA yields a protein of 27 kDa (lane 4). In the absence of β-mercaptoethanol, a product of 43 kDa was also observed (lane 5), suggesting intramolecular or intermolecular disulfide bridges and the formation of homodimers or heterodimers with other protein(s) present in the lysate.

FIGURE 4. Sequence comparison between human *HCaRG* and rat *HCaRG*. The deduced amino acid sequences of rat *HCaRG* (*rHCaRG*) and of human *HCaRG* (*hHCaRG*) are aligned. Identical amino acids are boxed while homologous amino acids are shaded. We calculated 80% homology between these 2 sequences. Analysis revealed homology to the EF-hand motif, with 8 out of the 10 most conserved amino acids (dashed box). Further analysis using the PROSEARCH database revealed 4 overlapping putative 'leucine zipper' consensus motifs (underlined). We also identified a nuclear receptor-binding domain (bold and italics).

FIGURE 5. Subcellular localisation of *HCaRG* in cultured cells. COS-7 cells were transfected with GFP-*HCaRG*. 24 h later, the cells were fixed and observed. Cells transfected with pEGFP vector alone show diffuse fluorescence (A) while cells transfected with pEGFP-*HCaRG* present nuclear fluorescence (B). Nuclear localization was confirmed by immunofluorescence on COS-7 cells transfected with pcDNA1/Neo-*HCaRG* (C), and by electron microscopy (D) on pituitary.

FIGURE 6. Tissue distribution of *HCaRG* mRNA. *A.* Comparison of *HCaRG* expression in fetal versus adult human organs. *HCaRG* mRNA is expressed less in all fetal tissues compared, particularly in the heart, kidney and liver (■ adult; ▨ fetal). *B.* Northern blot containing 2 µg of polyA$^+$ RNA from fetal and adult human hearts. HCaRG is more expressed in all regions of the adult heart (L: left, R: right). *C.* Comparison of *HCaRG* expression in adult human organs versus cancerous cell lines. *HCaRG* mRNA is expressed less in most cancerous cell lines compared. Lymphocyte (■normal; □Burkitt's lymphoma Raji; ▨ Burkitt's lymphoma Daudi). Leukocyte (■ normal; □ leukemia HL-60; ▨ leukemia K-562; ▥ leukemia MOLT-4). Rectum (■normal; □colorectal adenocarcinoma SW480). Lung (■ normal; □ lung carcinoma A549). *D.* Northern blot containing 20 µg of total RNA isolated from 3 different human tumours (T) and normal tissue (N) excised at the same operational site. *HCaRG* expression is decreased in brain, kidney and liver tumours.

FIGURE 7. *In situ* hybridization of *HCaRG* mRNA in the kidney and adrenal. *In situ* hybridization of *HCaRG* mRNA in the rat adrenal shows specific detection in the zona

fasciculata and medulla. Specific hybridization in the kidney is restricted to proximal tubules, contrasting with virtual absence in the glomeruli (G). (Upper panels: antisense probe, lower panels: sense probe).

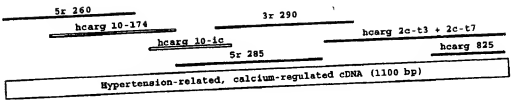
FIGURE 8. Analysis of kidney mRNA of *HCaRG* and *c-myc* obtained after ischemia and various periods of reperfusion. *A.* Dot blot of total RNA taken from the medulla of kidneys which underwent 60-min ischemia and reperfusion for various time periods (full lines) or from contralateral control kidneys (dotted lines). *HCaRG* mRNA declined rapidly to its lowest levels at 3 h and 6 h of reperfusion. It then increased steadily to exceed baseline at 48 h of reperfusion. In contrast, *c*-myc mRNA levels rose dramatically by 12 h and returned below *HCaRG* mRNA levels at 48 h of reperfusion. *B.* Representative northern blots of *HCaRG* and *c-myc* mRNA from the cortex of kidneys which underwent 60-min ischemia and 3 h, 6 h, 12 h, 24 h or 48 h (*HCaRG*) or 12 h or 24 h (*c-myc*) of reperfusion (I/R) or from contralateral control kidneys (C).

FIGURE 9. Characterization of stable cell lines. *A.* HEK293 cells transfected with pcDNA1/Neo or pcDNA1/Neo rat *HCaRG* were examined for expression of rat *HCaRG* by northern blot using rat *HCaRG* as a probe. Rat *HCaRG* was undetectable in cells transfected with the empty vector while different levels of expression were observed in cells tranfected with the vector expressing *HCaRG*. *B.* The levels of ectopic expression were determined by densitometric measurement and normalized to GAPDH.

FIGURE 10. *HCaRG* expression inhibits cell proliferation. *A.* Stable clones Neo1, Neo6, Neo Poly, *HCaRG8*, *HCaRG9*, and *HCaRG* Poly were plated at low density. For each time point,

triplicate plates were counted, and average cell number was recorded. The level of DNA synthesis was monitored by measuring [^{3}H] thymidine incorporation (*B*). Representative experiment performed in triplicate.

A



B

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                                  -131 GCACGAGCCACAGCCAGCTACCGCGGCTAGGTTCCTCCAGGTGCAGAGGGCG  -80
GTAAAGGCTTGGTTTGTATTTGTAATGCAACTGTGGTTAGGACCTTCTCTTCGGACTGGTCAAGAAACGGGAAGAAAGG          -1
ATG TCT GCT TTG GGG GCT GCA GCT CCA TAC TTG CAC CAT CCC GCT GAC AGT CAC AGT GGC             60
Met Ser Ala Leu Gly Ala Ala Ala Pro Tyr Leu His His Pro Ala Asp Ser His Ser Gly

CGG GTC AGT TTC CTG GGT TCC CAG CCC TCT CCA GAA GTG ACG GCC GTG GCT CAG CTC TTG           120
Arg Val Ser Phe Leu Gly Ser Gln Pro Ser Pro Glu Val Thr Ala Val Ala Gln Leu Leu

AAG GAC TTA GAC AGG AGC ACC TTC AGA AAG TTG TTG AAA CTT GTA GTC GGG GCC CTG CAT           180
Lys Asp Leu Asp Arg Ser Thr Phe Arg Lys Leu Leu Lys Leu Val Val Gly Ala Leu His

GGG AAA GAC TGC AGA GAA GCT GTG GAG CAA CTT GGT GCC AGC GCC AAC CTG TCA GAA GAG           240
Gly Lys Asp Cys Arg Glu Ala Val Glu Gln Leu Gly Ala Ser Ala Asn Leu Ser Glu Glu

CGT CTG GCC GTC CTG CTG GCG GGC ACA CAC ACC CTG CTC CAG CAG GCT CTC CGG CTG CCC           300
Arg Leu Ala Val Leu Leu Ala Gly Thr His Thr Leu Leu Gln Gln Ala Leu Arg Leu Pro

CCT GCT AGT CTA AAG CCA GAT GCC TTC CAG GAA GAG CTC CAG GAA CTT GGC ATT CCT CAG           360
Pro Ala Ser Leu Lys Pro Asp Ala Phe Gln Glu Glu Leu Gln Glu Leu Gly Ile Pro Gln

GAT CTA ATT GGA GAT TTG GCC AGT TTG GCA TTT GGG AGT CAA CGC CCT CTT CTC GAC TCT           420
Asp Leu Ile Gly Asp Leu Ala Ser Leu Ala Phe Gly Ser Gln Arg Pro Leu Leu Asp Ser

GTA GCC CAA CAG CAG GGA TCC TCG CTG CCT CAC GTG TCT TAC TTC CGG TGG CGG GTG GAC           480
Val Ala Gln Gln Gln Gly Ser Ser Leu Pro His Val Ser Tyr Phe Arg Trp Arg Val Asp

GTG GCC ATC TCA ACC AGC GCT CAG TCC CGC TCC CTG CAA CCG AGT GTT CTC ATG CAG CTG           540
Val Ala Ile Ser Thr Ser Ala Gln Ser Arg Ser Leu Gln Pro Ser Val Leu Met Gln Leu

AAG CTC ACA GAT GGA TCT GCA CAC CGC TTC GAG GTG CCC ATA GCC AAA TTT CAG GAG CTG           600
Lys Leu Thr Asp Gly Ser Ala His Arg Phe Glu Val Pro Ile Ala Lys Phe Gln Glu Leu

CGG TAC AGT GTA GCC TTG GTC CTT AAG GAG ATG GCA GAA CTG GAG AAG AAG TGT GAG CGC           660
Arg Tyr Ser Val Ala Leu Val Leu Lys Glu Met Ala Glu Leu Glu Lys Lys Cys Glu Arg

AAA CTG CAG GAC TGA CTGAACCCTGGTACTGTGGGTGCTGAAGCTGGTACCAGAACACAGCCCCCCACTGGTGA          734
Lys Leu Gln Asp TER

TGAGCCCAACTCCATTGAGGTCCTGCATGTGAGAACGTATTTTAAGTGAAAAGACAGCGGGACTTTCAGGTTTTGTTTT          813
ATGAGTCAACAGCTCGGCAGGGTGGCACAGTTTATAATCTCAGCCCTTGGAAGTCTGAGGCTGGAGAATGGGAAGTGTA          892
AGCTGGGCCTGGCTTTCATAGTGAGGCTCAGTGTCGAATTAAAGAGGTAAAGCAACTATTAAAAAAAAAAAAAAAA             969
```

FIGURE 1

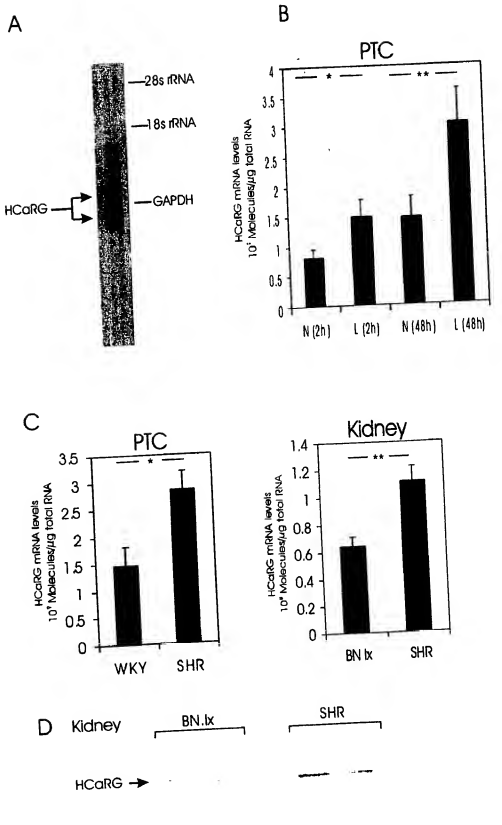

A
28s rRNA
18s rRNA
GAPDH
HCaRG
B
PTC
HCaRG mRNA levels
10³ Molecules/µg total RNA
N (2h)
L (2h)
N (48h)
L (48h)
C
PTC
WKY
SHR
Kidney
BN lx
SHR
D
Kidney
BN.lx
SHR
HCaRG

FIGURE 2

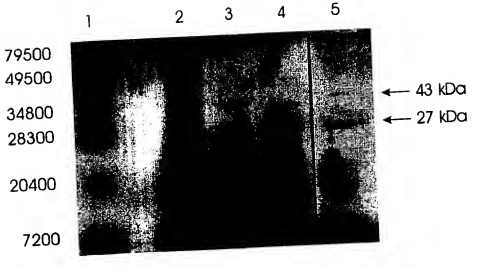


FIGURE 3

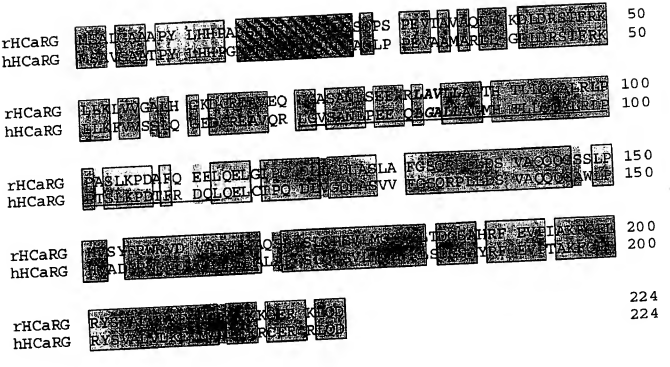


FIGURE 4

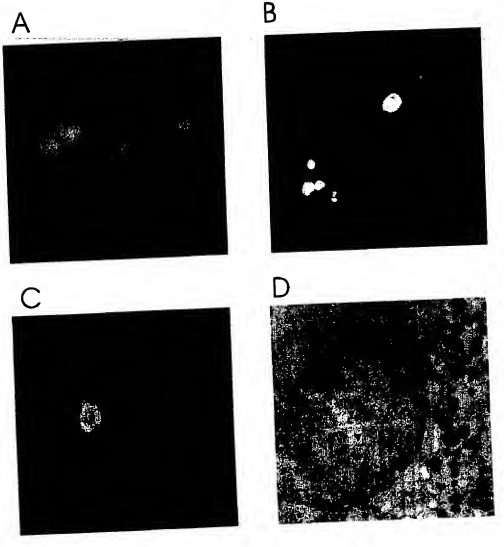
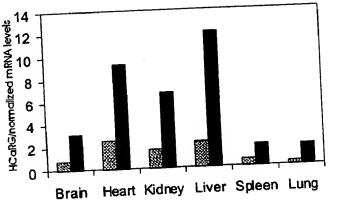


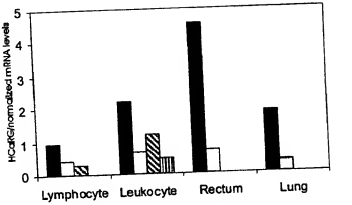
FIGURE 5

CA 02312266 2000-07-14

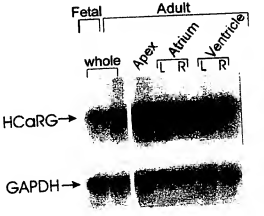
A Fetal/Adult



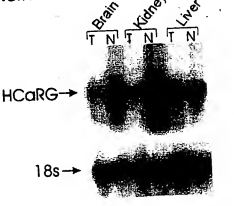
C Normal/Cancerous



B HEART



D Tumor/Normal



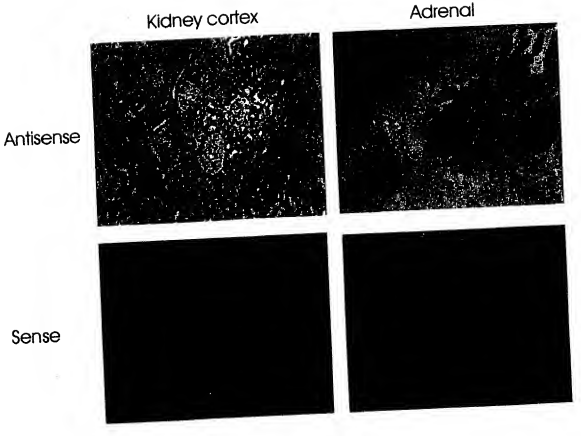
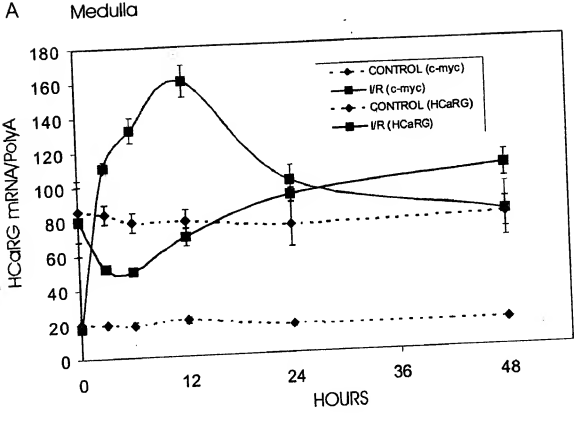
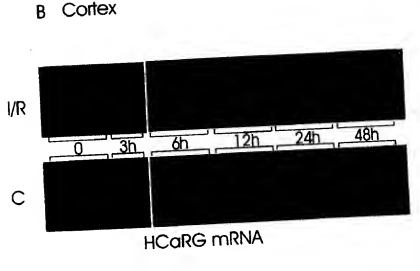


FIGURE 7


A
Medulla
HCaRG mRNA/PolyA
HOURS
CONTROL (c-myc)
I/R (c-myc)
CONTROL (HCaRG)
I/R (HCaRG)
0
20
40
60
80
100
120
140
160
180
0
12
24
36
48


B Cortex
I/R
C
0
3h
6h
12h
24h
48h
HCaRG mRNA

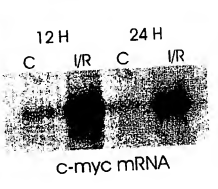
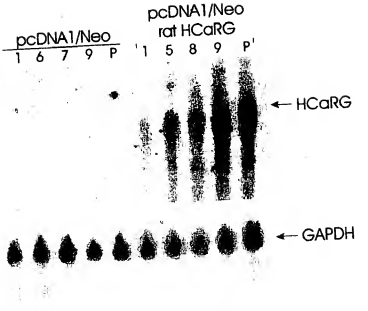

C Cortex
12 H
24 H
C
I/R
C
I/R
c-myc mRNA

FIGURE 8

A


pcDNA1/Neo
1 6 7 9 P
pcDNA1/Neo
rat HCaRG
1 5 8 9 P'
HCaRG
GAPDH

B

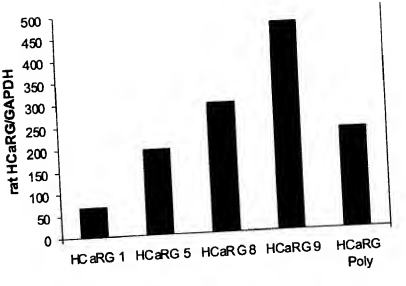
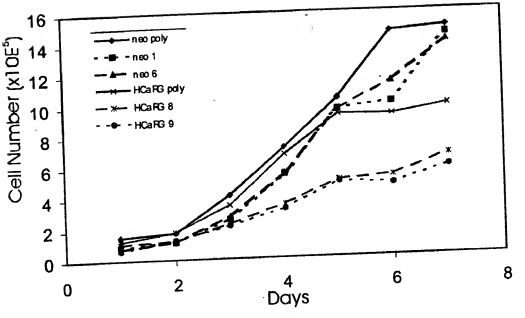

rat HCaRG/GAPDH
500
450
400
350
300
250
200
150
100
50
0
HCaRG 1
HCaRG 5
HCaRG 8
HCaRG 9
HCaRG Poly

FIGURE 9

A


Cell Number (x10E5)
16
14
12
10
8
6
4
2
0
0
2
4
6
8
Days
neo poly
neo 1
neo 6
HCaRG poly
HCaRG 8
HCaRG 9

B

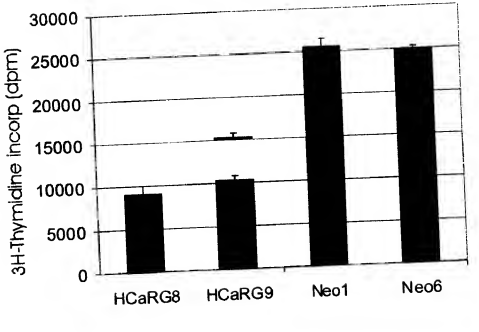

3H-Thymidine incorp (dpm)
30000
25000
20000
15000
10000
5000
0
HCaRG8
HCaRG9
Neo1
Neo6

FIGURE 10